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Rapid and Simple Diagnosis of the Two Common α_1-Proteinase Inhibitor Deficiency Alleles Pi*Z and Pi*S by DNA Analysis

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Summary: We describe a simple DNA-based method to assign the two common α_1-proteinase inhibitor (α_1-antitrypsin) deficiency alleles in the Pi-system (Pi*Z and Pi*S). Two sets of mutated primers are used in the polymerase chain reaction (PCR), followed by a restriction enzyme digest of the products. The mutated forward primers create a *Taq* I site only if the wildtype alleles (mostly M or subtypes) are present and not in the presence of the Pi*Z or Pi*S alleles. The reverse primers are mutated for an invariant *Taq* I site which serves as an internal control site in order to assure the completion of the restriction enzyme digest. The digested PCR products can be clearly resolved by 2.5% MetaPhore™-agarose gel electrophoresis. This simple PCR probing of the most common α_1-antiproteinase deficiency alleles can be routinely applied either to samples showing quantitatively decreased α_1-antiproteinase values in serum or to blood spots of *Guthrie* cards used for mass screening purposes. In addition, this method may provide the opportunity for a simple, rapid, and reliable prenatal diagnosis of α_1-antiproteinase deficiency in special cases.

Introduction

α_1-Antiproteinase (α_1-antitrypsin) is a M_r 52 000 glycoprotein mainly synthesized in hepatocytes and alveolar macrophages. It is an important protease inhibitor and its major physiological substrate is elastase, particularly in the lower respiratory tract (1). The α_1-antiproteinase gene, spanning 12 200 bases, is located on chromosome 14q31–32 (2). It is comprised of six introns and of three non-coding exons at the 5'-site followed by four coding exons (3). α_1-Antiproteinase shows considerable genetic variability. More than 90 different polymorphic alleles or variants are known (4). The most common allele is PiM1, which exists in two subtypes, M1A and M1V, characterized by an alanine or a valine at amino acid position 213, respectively (5). The majority of the polymorphic alleles are associated with normal concentrations of α_1-antiproteinase. Some deficiency alleles, however, are associated with low levels of α_1-antiproteinase in serum and can either be linked to liver disease and/or to early onset emphysema. The most common deficiency alleles are Pi*S and Pi*Z, which exhibit allele frequencies of 0.02–0.04 and 0.01–0.02, respectively, in European populations. Therefore, approximately 0.2% of the normal population are homozygous for either the Pi*S- or the Pi*Z-allele (1). The characterization of the different α_1-antiproteinase phenotypes is usually achieved by isoelectric focusing.

In 1984, *Long* et al. (6) sequenced the gene for the S variant at α_1-antiproteinase and, thus, opened the way to diagnosing α_1-antiproteinase deficiency alleles at the DNA level. In the past ten years a large number of different DNA-based methods to detect deficiency alleles of α_1-antiproteinase have been described: restriction fragment length polymorphism (RFLP) (7), allele specific oligonucleotide hybridisation (ASO) (7), allele specific amplification (18), direct sequencing (4), dual-colour detection by ligase-mediated analysis (9), temperature or denaturing gradient gel electrophoresis (TGGE, DGGE) (10), or PCR-mediated site-directed mutagenesis (11, 12, 13).

In this study, we describe a simple and rapid, non-radioactive, PCR-based method to detect Z and S mutations in the α_1-antiproteinase gene. The specific improvements in comparison with other hitherto published DNA-based techniques, in particular with respect to reliable quality control procedures, are discussed.

Materials and Methods

DNA preparation

Genomic DNA was prepared from 200 µl EDTA-treated whole blood by using the QIAamp Blood Kit from Qiagen No. 29104 (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Alternatively, DNA was prepared as described previously (14).

Polymerase chain reaction (PCR)

For PiZ-specific PCR the following mutated oligonucleotides were used: AAT-ZF (5'-GGCTGTGCTGACCATCGTC-3') and AAT-ZR

† H. C. deceased at January 17, 1994

(5'-AACTCTTCTTTAATGTCATCGA GG-3'). The A to T and the C to G substitutions are underlined. The primers were designed based on the cDNA sequence published by *Kurachi* et al. (15). For PiS-specific PCR the following mutated oligonucleotides were used: AAT-SF (5'-AGGGGAAACTACAGCACCTCG-3') and AAT-SR (5'-TGGGTACTGTTCTCCTCATCGAGCATG-3'). Both G to C substitutions are in italics and underlined. These primers were designed based on the genomic DNA sequence published by *Long* et al. (6). The PCR reactions were performed separately for each allele in 0.5 ml reaction tubes. The total reaction volume of 20 µl included 1 µl genomic DNA, 5 pmol of each primer, 1.25 U of *Taq* DNA polymerase (Boehringer-Mannheim, Germany), 200 µmol of dNTPs (Pharmacia, Freiburg, Germany), and 1.5 mmol/l magnesium chloride. Each sample was subjected to 38 amplification cycles of 45 s at 94 °C, 45 s at 53 °C, and 30 s at 72 °C on Hybaid's thermoreactor OmniGene. Initial denaturation was performed at 94 °C for 5 min and the final extension time was 5 min at 72 °C.

Restriction enzyme digest

The PCR products were digested with *Taq* I restriction enzyme (10 units/µl) purchased from Boehringer-Mannheim (Germany). One µl of *Taq* I restriction enzyme was added directly to the PCR tube and incubations were continued for 60 min at 65 °C.

Agarose gel electrophoresis

The entire volume of the digest was loaded onto a submarine gel and products were resolved in 2.5% MetaPhor™-agarose (Biozym, Hameln; Germany) at 100 V for two hours. The electrophoresis buffer was Tris-borate-EDTA (85 mmol/l Tris, 90 mmol/l boric acid, and 2 mmol/l EDTA) containing ethidium bromide (0.35 mg/l). Bands were visualized by UV fluorescence at 302 nm and photographed on Polaroid film. The DNA length standard V from Boehringer-Mannheim (Mannheim, Germany) was used as size marker.

Results

Detection of the Z-mutation

The Z-mutation is a single-base substitution in exon 5 of the normal M1 (Ala^{213}) allele causing a glutamate to

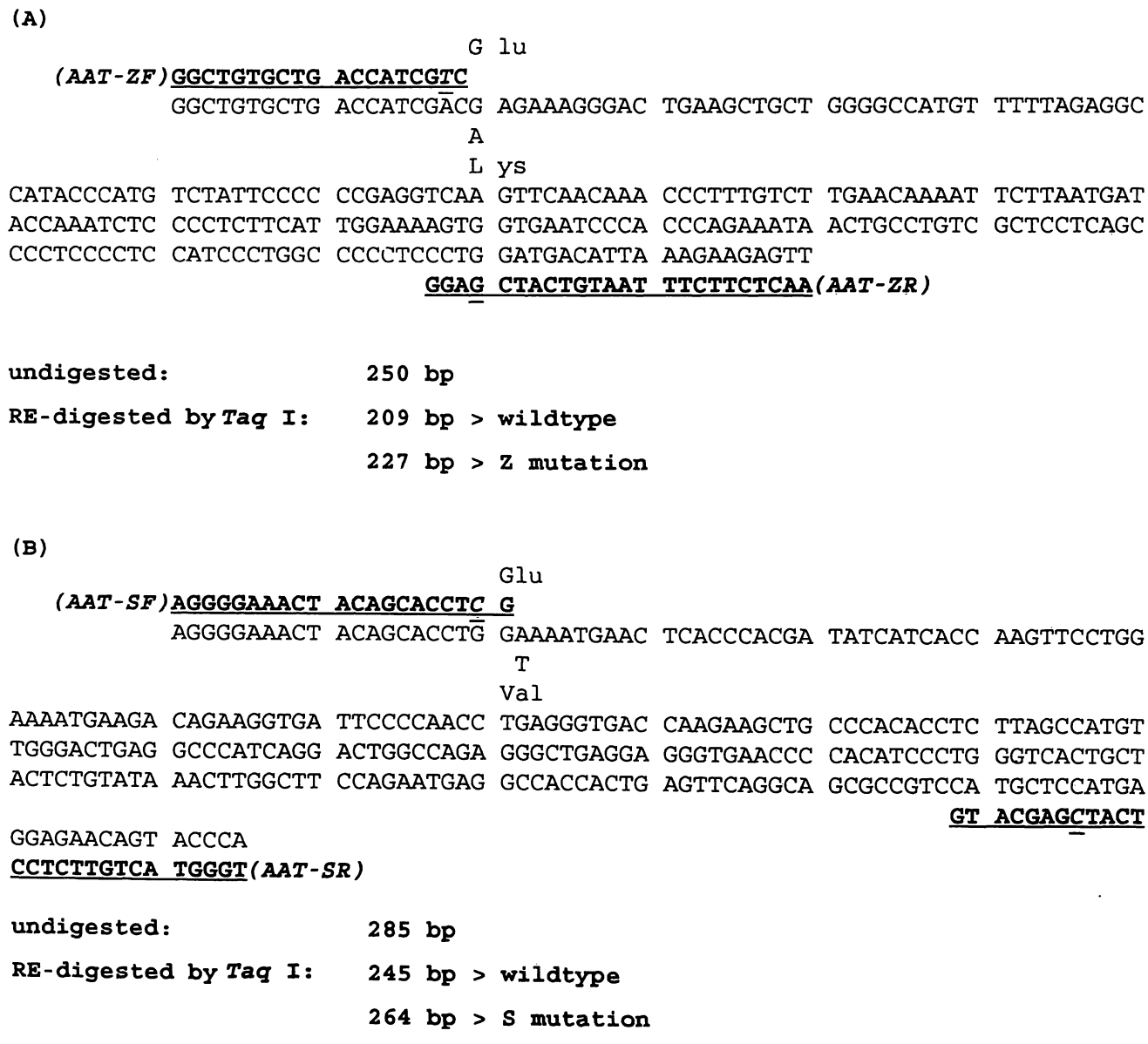

Fig. 1 Nucleotide sequences of the α_1-antiproteinase gene amplified by PCR and principle of the assay for detection of the Z mutation (A) and of the S mutation (B). The primer sequences are printed in bold letters and are underlined. Mutated nucleotides are in italics and doubly underlined. The size of undigested or *Taq* I restriction enzyme digested PCR products are indicated.

lysine substitution at amino acid position 342. The primer pairs, designed to amplify the sequence containing the Z mutation (Glu342Lys), produced a 250 base pair produced from part of exon 5 of the α_1-antiproteinase gene. The amplified sequence, including the site of the mutation and the primers, is depicted in figure 1 (A). The AAT-ZF primer introduces a diagnostic *Taq* I site only if the PCR product is derived from normal alleles (codon 342 GAG) and not in products from alleles bearing the Z mutation (codon 342 AAG). The presence or absence of this *Taq* I site makes it possible to distinguish between normal and Z mutation-bearing alleles after digestion with *Taq* I restriction enzyme followed by agarose gel electrophoresis. The AAT-ZR primer introduces an invariant *Taq* I site in all PCR products. This *Taq* I site serves as an internal control for completion of the restriction enzyme digest. Amplicons from patients with the homozygous PiZZ phenotype showed a single band of 227 bp (fig. 2, lane 5). Two bands of 209 base pairs and 227 base pairs were seen in PCR products from individuals with PiMZ phenotype (fig. 2, lane 4). Only a single band of 209 base pairs was seen in normal phenotypes, e. g. PiMM (fig. 2, lane 3). Partial digestion cannot account for the results since incomplete digestion would show an additional band of 250 base pairs.

Detection of the S-mutation

The S-mutation is a single base substitution in exon 3 causing a glutamate to valine substitution at amino acid position 264. The primers used to amplify the sequence that includes the S mutation (Glu264Val) yield a 285 base pairs fragment from part of exon 3 and part of intron 3 of the α_1-antiproteinase gene. The amplified sequence, including the site of the mutation and the primers, is depicted in figure 1 (B). In the same manner as for the detection of the Z-mutation, the AAT-SF primer introduces a diagnostic *Taq* I site only in the PCR product from normal alleles (codon 264 GAA) and not in that from alleles bearing the S-mutation (codon 264 GTA). Again the AAT-SR primer introduces an invariant *Taq* I site in all PCR products that serves as an internal control for the completion of the restriction enzyme digest. Two bands of 245 base pairs and 264 base pairs were seen in products derived from PiMS individuals (fig. 2, lane 7) whereas a single band of 245 base pairs was seen in the normal phenotype, e. g. PiMM (fig. 2, lane 8). Incomplete digestion would show an additional band of 285 base pairs.

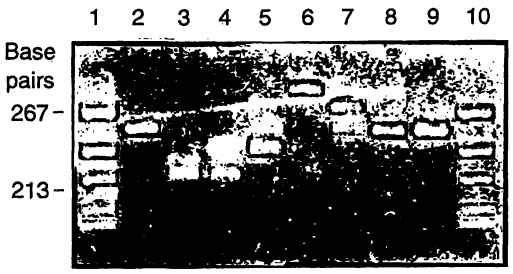

Fig. 2 Ethidium-bromide-stained 2.5% MetaPhor™-agarose gel showing the products of amplified normal and mutant alleles of α_1-antiproteinase. Base pairs are indicated at the left margin.
(1) and (10) DNA length standard V from Boehringer-Mannheim;
(2)–(5) reuslts of the Z-mutant typing: (2) undigested PCR product, (3) PiMM, (4) PiMZ, (5) PiZZ,
(6)–(9) results of the S-mutant typing: (6) undigested PCR product, (7) PiMS, (8) and (9) PiMM.

In a preliminary trial to evaluate the assay, we tested genomic DNA from 10 patients who showed decreased quantitative α_1-antiproteinase concentrations in serum (< 1.1 g/l) as assessed by nephelometric measurements with the Beckman Array system. Among those, 3 patients were found to be heterozygous for the Pi*Z-allele and 2 other patients heterozygous for the Pi*S-allele, respectively. Five patients did not bear either the Pi*Z or the Pi*S-allele. Examination of the patients' sera by isoelectric focusing yielded the same unambiguous results (data not shown). The 5 patients, showing decreased α_1-antiproteinase concentrations despite the absence of the Pi*Z- or Pi*S-allele, were characterized as carriers for common Pi*M alleles in the heterozygous state.

Discussion

Diagnosis of α_1-antiproteinase deficiency has previously been based on quantitative measurement of the α_1-antiproteinase concentration in serum followed by isoelectric focusing. In specialized laboratories isoelectric focusing is most reliable to assess the wide variety of disease-associated α_1-antiproteinase deficiency alleles. In the South German population, the combined frequencies of uncommon proteinase inhibitor phenotypes are approximately 7.5% (*S. Weidinger*, personal communication). Among these, the 5 phenotypes PiMS, PiMZ, PiSS, PiZZ, and PiSZ, leading to significantly reduced α_1-antiproteinase levels, comprise 85% of the total. Therefore, for practical purposes, the detection of these 5 phenotypes appears to be sufficient to account for reasonable diagnostic sensitivity.

We used PCR primer mediated mutagenesis to create convenient restriction enzyme recognition sites since the restriction enzyme digest is the most critical step in the characterization of the α_1-antiproteinase-specific PCR products and incomplete digestion would result in false positive results. Therefore, in order to ensure that successful *Taq* I restriction enzyme cleavage of PCR products has occured, we incorporated a second invariant *Taq* I restriction site as an internal control site for effective enzyme action. Alternative methods for detection of the Z and S mutations, such as allele-specific oligonucleotide hybridisation (ASO), allele-specific amplification (ASA), direct sequencing, ligase mediated analysis, temperature gradient gel electrophoresis, and

denaturing gradient gel electrophoresis are either time consuming or less reliable. Therefore, the availability of a simple and well controlled DNA test like this one is favoured, not only as a routine laboratory technique but also for prenatal diagnosis or for mass screening purposes.

Techniques similar to ours to detect the Z and S mutation of the α_1-antiproteinase gene have previously been proposed by *Dry* (11) and *Tazelaar* et al. (12). Our method, however, is faster because of shorter PCR cycle times and shorter restriction enzyme cleaving times. In addition, much more important is the fact that *Dry* (11) and *Tazelaar* et al. (12) used primers that do not incorporate control sites for restriction enzyme cleavage. *Andresen* et al. (13) first designed primers that include control sites for *Taq* I and *Xmn* I restriction enzymes for the detection of PiZ and PiS, respectively. Our technique is somewhat faster and uses 2.5% MetaPhore™-agarose instead of 16% polyacrylamide for electrophoretic resolution of PCR products.

The conditions for DNA extraction, PCR, and agarose gel electrophoresis are designed so that they can be used in a very similar manner for other routine diagnostic DNA tests. These include the diagnosis of the most common genetic defect (Factor V Leiden) underlying activated protein C resistance in patients with thrombophilia (16), the apolipoprotein E genotyping (17), and the diagnosis of apolipoprotein B-100 mutation Arg3500Gln (*Braun*, unpublished). We consider the potential to run several different diagnostic assays under defined and similar conditions as an important step to improve the quality in processing DNA-based tests in clinical chemistry. In conclusion, our method for identifying the most relevant α_1-antiproteinase deficiency alleles is simple, fast and satisfies the quality control features necessary for application in routine laboratory settings.

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A Principle of Quality Assessment Using a Competitive Polymerase Chain Reaction Assay for the Detection of *Chlamydia trachomatis* in Cervical Specimens

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Dedicated to Prof. Dr. Dr. J. Büttner on the occasion of his 65th birthday

Summary: The polymerase chain reaction (PCR)-based identification of *Chlamydia trachomatis* in clinical specimens should include a built-in control to assess the quality of the whole assay from DNA isolation to detection. For this purpose we established a competitive PCR assay with the following design. A 215 base-pair DNA fragment from a *Chlamydia trachomatis* plasmid sequence was amplified in a polymerase chain reaction. An internal control DNA was coamplified in the same reaction. The differentiation between the amplified *C. trachomatis* DNA and the internal control is based on hybridisation against two different probes using Enzymun Test® DNA detection (Boehringer Mannheim). The internal control capture probe recognizes an additional 20 base-pair DNA sequence in the competitor DNA construct. Cervical swabs from 65 *C. trachomatis* positive patients were used. We examined the influence of different DNA isolation methods on the sensitivity of the assay. Detection of *C. trachomatis* from positive cervical swabs was compared using PCR and competitive PCR assays. The advantage of the competitive assay was a better assessment of reduced sensitivity arising from inhibitory effects or mistakes during the DNA preparation or amplification.

Introduction

Use of the polymerase chain reaction for identification of *Chlamydia trachomatis* in different specimens, like urine or cervical swabs, has attained a state of the art in recent years (1–4). In numerous publications, the diagnostic tool of PCR has been compared with the "gold standard", the culture of *Chlamydia trachomatis* (5–9). These comparisons showed that PCR has better diagnostic sensitivity and comparable specificity. False-positive results were generally absent, and could be eliminated by using suitable assay conditions (10–12). Exclusion of false-negative results, however, has hitherto been largely neglected. Negative results, especially in the detection of obligate pathogenic infectious agents like *Chlamydia trachomatis*, should be validated with suitable control materials. For this purpose, the control materials are generally bacterial control strains, which are prepared and used for amplification of the target DNA in additional reactions. For this external control of the DNA isolation and amplification plasmids with an insertion in the target DNA region have also been used (13). Other authors have described control reactions using a second primer pair for the coamplification of an additional DNA fragment of human origin (14, 15). But these materials do not allow a definite control of each sample from DNA preparation to detection.

For an optimized quality assessment and the avoidance of false-negative results, a coamplification of a competitor DNA is an effective tool (16–18). We used a plasmid DNA with an insertion in a modified part of the target DNA region of the *Chlamydia trachomatis* cryptic plasmid. This region was shown to be most sensitive for identification of the infectious agent in comparison to other genomic regions like rRNA genes or the major outer membrane protein (19, 20). The internal control DNA was added to all patient samples and coamplified with the same pair. We compared the competitive PCR with a regular PCR assay on an automated analyser system, using different DNA isolation techniques.

Materials and Methods

Samples

Samples were collected from patients of various gynaecologic practices between November 1994 and November 1995. Cervical swabs were collected with a dacron-tipped swab sets (Becton Dickinson). The samples were placed in 500 µl lysis buffer containing 10 g/l Triton-X 100 in physiological saline solution in a sterile reaction tube (Eppendorf, 2 ml), resuspended and stored at 4–8 °C until needed.

DNA isolation

Four µl of a tRNA solution (10 g/l) (Boehringer Mannheim) were added to 200 µl of the specimen in lysis buffer. For the competitive PCR the tRNA solution contained an additional 2 µg/l of a competitor DNA. The samples were extracted with an equal volume of phenol/chloroform (1 + 1), then with chloroform, and finally precipitated with a threefold volume of ethanol. The sedimented DNA was washed with 500 µl ethanol (75 g/l), dried and dissolved in 100 µl H_2O. DNA was also isolated using commercial kits like

QIAmp Blood kit including Qiagen Protease, QIAEX II Gel Extraction kit (Qiagen), High Pure PCR Template Preparation kit including Proteinase K (Boehringer Mannheim), and Dynabeads DNA DIREKT kit (Dynal). Preparation protocols were performed according to manufacturers' instructions.

Implementation of the polymerase chain reaction

PCR was performed as described by *Saiki* et al. with the following modifications (21). Ten µl of the sample treated as described above were added to 40 µl of the PCR-mix (PCR-mix: see below) in a 0.5 ml Eppendorf tube. The PCR was carried out with the primer pair PCL1/PCL3 in a thermocycler (Landgraf, MWG) for 35 cycles. Each cycle consisted of a denaturation step at 94 °C for 60 seconds, annealing at 55 °C for 60 seconds and elongation at 72 °C for 60 seconds.

The oligonucleotide sequences were:

PCL1: 5'-CTG TAA CAA CAA GTC AGG TTG CGC-3',
(nucleotides 202–225, (22))

PCL3: 5'-GTA CTA GAG GAC TTA CCT CTT CCC-3',
(nucleotides 416–393, (22))

PCR-mix (50 µl total volume): 0.2 mmol/l dNTPs (Boehringer Mannheim), 1 µmol/l each of the oligonucleotides (PCL1, PCL3) HPLC grade (Boehringer Mannheim), 2 µl PCR buffer (Boehringer Mannheim), 2 U *Taq* polymerase[1]) (Boehringer Mannheim), ad 40 µl H_2O.

For the immunochemical detection the DNA was labelled with digoxigenin-11-2'-deoxyuridine-5'-triphosphate, which was added to the deoxynucleoside-triphosphate mixture in the ratio 18 : 1 (dTTP/Dig-11-dUTP).

Immunochemical detection of DNA fragments

Fourty µl of the PCR-amplified and digoxigenin-labelled sample were added to 360 µl denaturing reagent (50 mmol/l NaOH) and pipetted into sample cups of an ES 300 analyser. The reaction programme was started as described in the Enzymun Test® DNA Detection instructions (Boehringer Mannheim). The following reagents were also necessary: Biotinylated capture probes were added to different hybridisation solutions (phosphate buffer, pH 6.5) to a final concentration of 90 µg/l.

Capture probe (*C. trachomatis*): 5' Biotin-CAC TTT GTC TCG ATG AGA GAC 3'
(nucleotides 374–354, (22))

Capture probe (internal control): 5' Biotin-GAT GCT GTC AGC ACT CTG AG 3'

C. trachomatis-specific DNA and the internal control DNA were detected by binding of the biotin-labelled nucleic acids to the streptavidin solid phase (Enzymun Test® Streptavidin Tubes, Boehringer Mannheim) in two different reactions. A peroxidase-conjugated anti-digoxigenin antibody was added to label the digoxigenin. The complex was then visualized by addition of the chromogen di-ammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate) (ABTS); the absorption of the resulting green colour was measured at 422 nm. Positive samples were determined by a cut-off value (cut-off = 2 × $E_{neg.}$, but > 0.1).

[1]) Enzymes:
Taq polymerase: DNA polymerase EC 2.7.7.7 from *Thermus aquaticus*
*Eco*RI: restriction endonuclease from *E. coli*
*Hind*III: restriction endonuclease from *Haemophilus influenzae* Rd
*Bam*HI: restriction endonuclease from *Bacillus amyloliquefaciens* H

Generation of the internal control DNA

The whole target DNA region was amplified in two different polymerase chain reactions under standard conditions (21) with the primer pairs PIK1E/PIK3B and PIK2H/PIK4B. The procedure was as described previously (17).

The sequences were:

PIK1E: 5'-TAA *GAA TTC* ACA TTT CCT ATC AGC TTA ATG GAA G-3'
(nucleotides 117–141, (22))

PIK2H: 5'-TAA *AAG CTT* GCA CGT TCT CTC AAG CAG GAC-3'
(nucleotides 696–676, (22))

PIK3B: 5'-TAA *GGA TCC* AAG TGT GTG TTC TTA TTG TTC-3'
(nucleotides 370–390, (22))

PIK4B: 5'-TAA *GGA TCC* CTA GAG AGA CAG GAA ATA CGC ATG-3'
(nucleotides 363–340, (22))

The amplified products were gel purified using Sephaglas-Kit (Pharmacia) and cleaved with *Eco*RI[1]) and *Bam*HI[1]) (PIK1E/PIK4B), with *Hind*III[1]) and *Bam*HI[1]) (PIK2H/PIK3B) and ligated with *Eco*RI/*Hind*III-digested vector pUCBM20 (Boehringer Mannheim). Competent bacterial strain *E. coli* C600 was transformed with the recombinant plasmid DNA (23).

The plasmid DNA of a positive clone was purified in a large scale plasmid preparation. One µg plasmid DNA was cut with the restriction endonuclease *Bam*H1, gel purified and dephosphorylated. Further a 26 base-pair DNA fragment was ligated with this vector. For this purpose we used the following oligonucleotides:

PIK: 5'-AAA AGG ATC CGA TGC TGT CAG CAC TCT GAG GGA TCC AAA A-3'

PIKrev: 5'-TTT TGG ATC CCT CAG AGT GCT GAC AGC ATC GGA TCC TTT T-3'

Two-hundred ng of the oligonucleotides PIK and PIKrev were hybridized in a total volume of 20 µl in 1 mmol/l 2-mercaptoethanol, 5 mmol/l $MgCl_2$, 100 mmol/l NaCl and 10 mmol/l Tris-HCl, pH 8.0 for one hour at 37 °C. The double stranded oligonucleotide was digested with *Bam* HI, extracted with phenol/chloroform, chloroform and dialysed against Tris-EDTA buffer using microdialysis membranes (Millipore). Twenty ng vector DNA were ligated with 1 ng of the digested double stranded oligonucleotide and amplified in the *E. coli* strain C600. After purification in a large scale plasmid preparation the DNA was ready to use for the PCR assay.

Plasmid preparation

Plasmid preparation was performed according to the instructions of Qiagen Plasmid Maxi Kit (Qiagen). The DNA was further extracted with phenol/chloroform, then precipitated with ethanol after RNase treatment of the plasmid DNA. The preparation was checked on an agarose gel. Agarose gel electrophoresis was performed on 20 g/l agarose gels in Tris-borate buffer containing 89 mmol/l Tris-borate, 2 mmol/l EDTA, 400 µg/l ethidium bromide, pH 8.0 (23). Electrophoresis was carried out for 45 minutes with 10 V/cm in Tris-borate buffer. The DNA bands were detected with an UV transilluminator at 312 nm (23). The concentration of the DNA was determined by measuring the absorbance at 260 nm. An absorbance of 1.0 corresponds to approximately 50 mg/l double-stranded DNA (23).

Results

Cloning the internal control DNA in *E. coli*

C. trachomatis DNA isolated from cervical specimens was amplified in two separate PCR reactions using the

primer pairs PIK1E/PIK4B and PIK3B/PIK2H. All primers contained additional recognition sites for restriction endonucleases. The joining of the two resulting DNA fragments via the *Bam*HI restriction sites of PIK3B and PIK4B generated four substitutions in the original sequence. More bases were exchanged by introducing three mismatches next to the cloning site of oligonucleotide PIK4B. After ligation with pUC BM20 and amplification in *E. coli* a 26 pase-pair DNA fragment was introduced in the *Bam*HI cloning site of the recombinant plasmid. The purified DNA was used as an internal control in the competitive assay.

Checking the assay conditions with internal control DNA

The application of a competitive PCR assay may result in a decreased amplification rate of the target DNA fragment. Therefore we determined the minimal quantity of competitor DNA, which can be coamplified in the PCR assay. Table 1 shows the results of this investigation. The internal control DNA was diluted from 10^{-8} g to 10^{-15} g per assay. From each dilution eight amplifications were performed and the products were detected with internal control capture probe using Enzymun Test® DNA Detection. One pg internal control DNA per 50 µl total volume in the reaction mixture was the lowest concentration resulting in a constant absorbance at 422 nm, as indicated by the increasing coefficients of variation at lower quantities of internal control DNA.

The effect of DNA isolation methods on the reliability of the assay

We studied the effect of several DNA isolation methods on the reliability of the detection of internal control and target DNA. Table 2 shows the results of these comparisons. In part (A) the quality of the preparation of 1 pg competitor plasmid DNA was tested using five different isolation methods. Four methods used commercial kits. Only phenol/chloroform extraction and two kits using protease digestion and spin column purification resulted in coefficients of variation lower than 10% (n = 8) for the measured absorbance values. Using these three methods, a pool of *C. trachomatis* positive swabs, resuspended in lysis buffer, were coprepared with the competitor DNA. In all cases we found coefficients of variation lower than five percent for the absorbances of amplified *C. trachomatis* and competitor DNA.

Competitive PCR versus PCR

For reliable identification of *Chlamydia trachomatis* in cervical specimens it was necessary to examine the diagnostic sensitivity of the described assay conditions. Sixty-five PCR-positive samples were determined with the different PCR assays as shown in figure 1. All assays included a hybridisation step, and a biotinylated probe was used in the competitive PCR (a) and PCR (b). There was good discrimination between the cut-off values and positive results, although the comparison of these methods (c) shows a tendency to lower absorbances in the competitive PCR, which is expressed by the regression equation 2.19 + 0.62x. These differences may depend on the ratio target DNA/competitor DNA, which is shown in figure 2, where several dilutions of pooled *C. trachomatis* DNA isolated from cervical swabs were examined in a PCR and a competitive PCR assay.

The efficacy of this system in revealing inhibitory substances was tested using different concentrations of an inhibitor solution (commercial washing buffer from a DNA preparation kit) as shown in figure 2. A decrease of the internal control absorbances revealed an inhibitory effect in each case (see fig. 2b, c).

In conclusion we found a minimal quantity of 1 pg competitor DNA, which can be coamplified with the *Chlamydia trachomatis* DNA isolated from cervical swabs. Using suitable DNA isolation methods like phenol/chloroform extraction or spin column purification, the coefficients of variation for values of internal control DNA were lower than 10%. A tendency to lower absorbance values after competitive PCR was shown, but the diagnostic sensitivity for the identification of *Chlamydia trachomatis* in cervical swabs was adequate.

Discussion

The use of PCR in routine diagnosis requires extensive quality control. There are numerous reports on the use

Tab. 1 Determining the minimum quantity of internal control DNA in the assay.
Internal control DNA was diluted in several steps (10^{-8} g–10^{-15} g/assay) and amplified by PCR using the primer pair PCL1/PCL3 (see materials and methods). From each dilution eight amplifications were performed; products were hybridized with the internal control capture probe. Mean values, standard deviations and coefficients of variation are shown for the measured absorbances at 422 nm.

Quantity [g]	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	10^{-13}	10^{-14}
Mean value (n = 8)	6.51	6.47	6.43	5.85	5.99	4.98	3.24
Standard deviation	0.06	0.07	0.06	0.14	0.12	0.50	1.21
Coefficient of variation [%]	1	1	1	2	2	10	37

Tab. 2 Effect of DNA preparation technique on the reliability of the PCR assay.
(A) 1 pg internal control DNA was treated according the protocols of five DNA isolation methods. (B) Samples from a pool of *C. trachomatis*-positive swabs in a lysis buffer were coprepared with 1 pg of the competitor DNA.
Mean values for absorbances at 422 nm after hybridisation with internal control capture probe (IC), *C. trachomatis* specific capture probe (PC), standard deviations (SD) and coefficients of variation (CV) are listed (n = 8).

		$\bar{x}$ (n = 8)	SD	CV [%]
A) Negative control with 1 pg internal control DNA (IC)				
Dynabeads DNA DIRECT Kit	IC	4.49	0.86	19
High Pure PCR Template Preparation Kit	IC	5.61	0.18	3
QIAamp Blood Kit	IC	4.75	0.50	10
QIAEX II Gel Extraction Kit	IC	2.57	1.01	39
Phenol/Chloroform extraction	IC	4.63	0.21	5
B) Positive pool (PC) with 1 pg internal control DNA (IC)				
High Pure PCR Template Preparation Kit	PC	4.54	0.09	2
	IC	5.58	0.14	3
QIAamp Blood Kit	PC	4.47	0.13	3
	IC	5.85	0.12	2
Phenol/Chloroform extraction	PC	4.44	0.07	2
	IC	5.31	0.17	3

of PCR in the diagnosis of *Chlamydia trachomatis* from different specimens (1–3, 6, 7) using commercial and in-house assays. Some authors have considered the reduction of false-positive results by using the commercial Amplicor kit (Roche) with the uracil N-glycosylase system for avoidance of contaminations (10–12). Suitable control mechanisms for detecting false-negative results were neglected.

The competitive PCR assay is an effective tool for the assessment of reduced sensitivity. The advantage of this method is the coamplification of target and competitor DNA with the same primer pair to allow a definite control of all reactions, with an option for a semiquantification of the PCR products. Both DNA sequences are identical, except for a small DNA insertion in the internal control DNA, which is required for separate identification of the competitor. Because of the modular design of the sompetitor, a time-saving co-detection of internal controls from different assay types is possible in the daily routine.

Inadequate DNA preparations, insufficient amplifications, or inhibitory effects (17, 24, 25) may be responsible for a reduced sensitivity. Our results suggest the following assay design for an effective recognition of false-negative results:

Design of the assay

Each run consists of the patient samples and negative controls, which are prepared together with the competitor DNA. In the case of negative results for *C. tracho-*

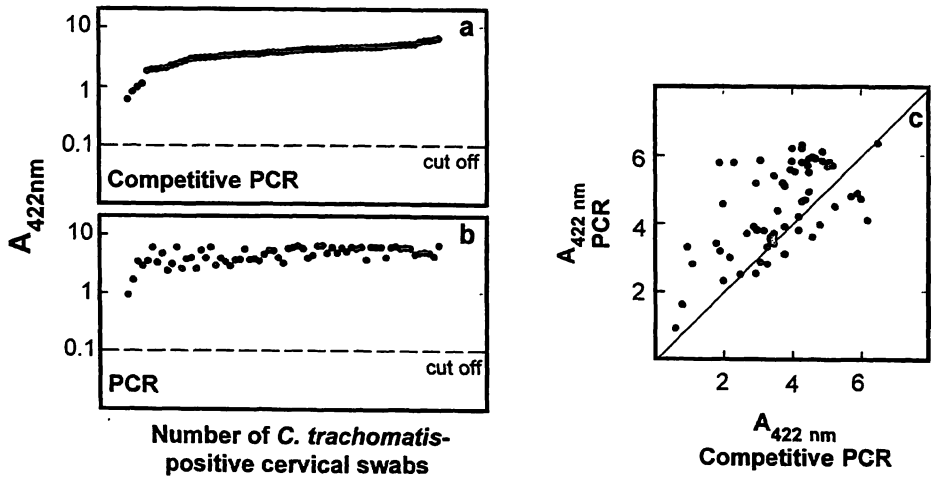

Fig. 1 Comparison of PCR and competitive PCR-based detection of *C. trachomatis* from cervical swabs.
Sixty-five *C. trachomatis* positive samples were analysed using competitive PCR (a) and PCR (b). Shown are absorbances at 422 nm versus the numbers of samples. The values under (a) and (b) are sorted pairwise in ascending order for the values under (b). Cut off values are indicated with a dotted line.
Under (c) a comparison of PCR versus competitive PCR of the 65 samples is shown (y = 2.19 + 0.62x, r = 0.62).

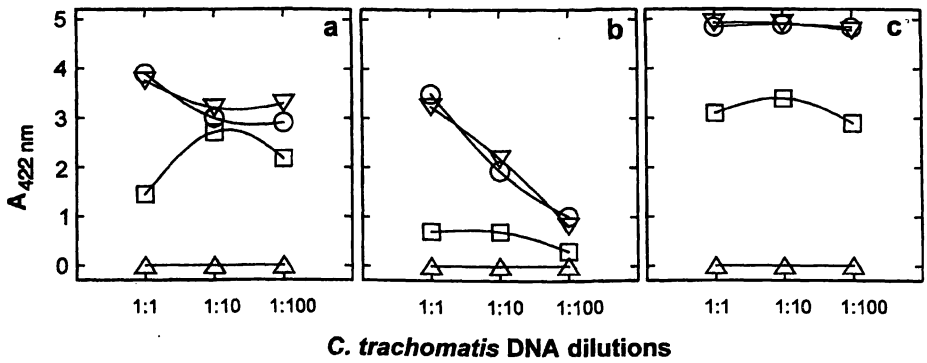

Fig. 2 Competitive PCR and inhibitory effects.
DNA isolated from *C. trachomatis* positive cervical swabs was pooled, diluted (1 : 10, 1 : 100) and amplified using PCR (a) and competitive PCR [*C. trachomatis* DNA (b), internal control DNA (c)]. Shown are absorbances at 422 nm versus the diluted samples. To demonstrate the advantage of a competitor DNA for quality assessment, different concentrations of an inhibitor solution (e. g. washing buffer (High Pure PCR Template Preparation Kit, Boehringer Mannheim)) were added to the PCR-Mix:
(o) no inhibitor, (▽) 1% inhibitor solution per assay, (□) 2.5% inhibitor solution per assay, (△) 5% inhibitor solution per assay.

matis the absorbance values for the amplified competitor DNA should be compared with those of the negative control competitors. Differences of more than three standard deviations of an inter-assay precision (or 10 percent in comparison with the control) will indicate a reduced sensitivity. In such cases the assay should be repeated starting with a new DNA preparation. Furthermore we propose a control measurement for all absorbances of amplified *C. trachomatis* DNA between $A_{422\,nm}$ 0.1–1.0 (see fig. 1a).

Using the described assay design it will be possible to assess the quality of each sample preparation in an assay. It may be possible to avoid false-positive results by using uracil N-glycosylase to diminish the measured absorbances. But the uracil N-glycosylase system provides insufficient protection, because it is directed only at amplicon contaminations. Our advice is to obey general guidelines for the avoidance of contaminations (e. g. separation of working areas) (26).

In summary it is possible to reduce false-positive results by rigorously following the rules for avoiding contamination. False-negative results, which arise from inadequacies in any preparation, can be revealed using the proposed competitive PCR assay.

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Osteocalcin Production In Vivo and In Vitro after 1,25-Dihydroxycholecalciferol Stimulation Comparison of Different Assays

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Summary: The study was designed to assess the sensitivity of three commercial assays (which differ in methodology, standard and antibodies) for osteocalcin, used for detecting changes in osteocalcin secretion induced by calcitriol (1,25-dihydroxycholecalciferol) in vivo and in vitro. Osteocalcin levels were determined in serum samples of 10 osteoporotic women after short term calcitriol treatment, and in the culture medium of human osteoblast-like cells (n = 22) after 48 h calcitriol exposure. All assays displayed similar sensitivity in detecting osteocalcin production in vivo after a 1 μg daily dose of calcitriol. A novel IRMA (CIS), claimed to detect intact osteocalcin, showed higher osteocalcin values than the other assays, and in vitro showed the best sensitivity; it provides an appropriate index of the osteocalcin synthetic activity of cultured human osteoblasts.

Introduction

Osteocalcin (bone gla protein) is a major bone matrix non-collagenous protein. It is a γ-carboxyglutamic acid-containing protein of 49 amino acids, with a relative molecular mass of M_r 5800. It is synthesized by bone forming cells and, in small amounts, by odontoblasts. Its synthesis is vitamin K dependent and markedly stimulated by calcitriol (1,25-dihydroxycholecalciferol) (1). Blood osteocalcin levels reflect new cellular synthesis, thereby serving as an index of the total body bone formation (2). Nevertheless, some osteocalcin is released from bone matrix during bone resorption (3). The determination of circulating osteocalcin is therefore of clinical interest and has become widely used to discriminate patients affected by different metabolic bone diseases (4–6). However, in a single clinical entity such as osteoporosis, a great variability in serum osteocalcin is observed. This was ascribed either to the heterogeneity of the disease (7) or to the differences of analytical methods and preanalytical factors (8). Owing to such variability, the determination of osteocalcin levels in a single osteoporotic patient has limited clinical relevance. The same might not be true when monitoring specific metabolic bone modifications or treatments where the changes in osteocalcin levels, independently of the absolute value, might have a clinical relevance. However, the sensitivity of different assays in detecting changes in osteocalcin levels following specific metabolic bone modifications or treatment has not been fully determined.

Therefore, we designed the following study to assess the sensitivity of three different commercial assays for serum osteocalcin, used for detecting changes in osteocalcin production induced in vivo and in vitro by a physiological stimulus. For this purpose, we performed sequential measurements of serum osteocalcin in osteoporotic patients during and after oral administration of calcitriol for 7 days, and we measured osteocalcin production in cultures of human osteoblast-like cells after exposure to calcitriol for 48 h.

Materials and Methods

In vivo

Ten women (mean age 71 ± 9 years) were admitted to the study after they had given their oral consent according with the procedures of our Ethical Committee. All women had a low bone mass characterized by a T-score, which is the number of standard deviations below the mean value of bone mineral density of young adult healthy women, ≥ 2 SD as assessed by dual energy X-ray absorptiometry (QDR 1000, Hologic, Waltham, MA, USA). Thus the patients were defined osteopenic according to the WHO panel (9). None of the subjects was affected by any other disease affecting bone metabolism, and none was under treatment with active bone agents. Their creatinine clearance was ≥ 50 ml/min. Creatinine was measured by the *Jaffe* method on the Hitachi 747 (Boehringer, Mannheim, Germany).

Oral daily doses of 0.5 μg or 1 μg of calcitriol (Rocaltrol, Roche, Basel, Switzerland) were administered for 7 days to two groups of five women. For three days before starting the administration of calcitriol the patients consumed a normal calcium (800 mg/d) and low sodium (100 mmol/d) diet. During the 7 days of treatment the calcium content of the diet was reduced to 600 mg/d. Fasting blood samples for osteocalcin determination were obtained immediately before, and 2, 4, 8 and 30 days from the beginning of treatment.

In vitro

Bone cells were established in culture by a modification of the *Gehron Robey & Termine* procedure (10) from trabecular bone samples obtained from waste materials during orthopaedic surgery for degenerative diseases or traumatic fractures of the femoral neck requiring osteotomy procedures. None of the patients (60–80 years old) submitted to surgery had any metabolic or malignant bone disease.

Briefly, the trabecular bone was cut into small pieces (2 × 2 × 2 mm) and washed thoroughly with commercial standardized *Joklik*'s modified MEM (Eurobio, Les Ulis, France) serum free medium to remove non adherent marrow cells. The bone pieces were incubated with the same medium containing 0.5 g/l collagenase (type IV, Sigma, St. Louis, MO, USA) at 37 °C for 30 minutes with rotation. The collagenase digestion was stopped by adding *Iscove*'s modified *Dulbecco*'s medium (Eurobio, Les Ulis, France) containing foetal bovine serum, volume fraction 0.2 (HyClone, Logan, UT, USA). The bone pieces (8–10 for each patient) were then placed in 25 cm^2 flasks and cultured in *Iscove*'s modified medium containing foetal bovine serum, volume fraction 0.2, 10^5 U/l penicillin, 100 mg/l streptomycin, 50 000 U/l mycostatin and 0.25 mg/l amphotericin B. Cells began to migrate within 1 week and reached confluence after 1 month. At this stage, wells were trypsinized and plated in 24 multiwell plates. Culture medium was changed every 2–3 days. In order to reduce the possibility of phenotype changes, all the cells were used at the first passage. Cells at confluence were divided into two groups, matched for donors: one group was incubated for 48 h with serum-free fresh medium alone, and the other with serum-free fresh medium supplemented with 10 nmol/l calcitriol (Hoffmann-La Roche, Basel, Switzerland).

Assays

For osteocalcin quantification three immunoassays were used: two isotopic and one non-isotopic. One method was a non-competitive immunoradiometric assay (IRMA), the other two were based on competitive methodology (RIA and enzyme immunoassay, EIA).

The IRMA is Elsa-osteo-nat (CIS, Gif-sur-Yvette, France) and utilizes two monoclonal antibodies against human osteocalcin. Purified human bone protein is used as standard calibrator. The antibody linked to the solid phase is directed against the 43–49 fragment whereas the antibody against the 5–13 fragment of the molecule is labelled with ^{125}I; the reported sensitivity is 0.3 µg/l.

The RIA is OSTK-PR (CIS, Gif-sur-Yvette, France). The principle of the assay is based on competition between ^{125}I-labelled osteocalcin and osteocalcin contained in standards or samples to be assayed for a fixed and limited number of antibody binding sites. The separation of bound and free fraction is based on the use of a immunoprecipitating reagent containing polyethylene glycol, sodium azide, sheep anti-rabbit immunoglobulins and non-immunized rabbit immunoglobulins. The antiserum is obtained from rabbits after bovine osteocalcin injections. The detection limit of the method is reported as 0.5 µg/l. The antibody specificity is not detailed.

The EIA is produced by DAKO (Glostrup, Denmark). The Dako osteocalcin ELISA is a competitive assay in which samples and biotinylated osteocalcin are incubated simultaneously in antibody-coated microwells. Highly purified bovine osteocalcin is used as a standard; polyclonal antibodies, directed against the COOH-terminus of the molecule are used. Osteocalcin standard and patient specimens are premixed with biotinylated osteocalcin in white microwells precoated with anti-osteocalcin. Following the first washing stage, peroxidase-conjugated streptavidin is added to the optically clear microwells. Following a second washing stage, the substrate (tetramethylbenzidine, in stabilized H_2O_2 solution) is added and the reaction is stopped with 2 mol/l H_2SO_4; absorbances are measured at 450 nm. The method sensitivity is then 0.3 µg/l.

The radioactivity of the standards and samples in the RIA and IRMA was measured in a γ-counter (MDA 512, Kontron, Milan, Italy).

The absorbances of EIA standards and samples were measured by an automatic EIA-reader (ETI system, Sorin, Saluggia, Italy). Internal controls were established for all measurements. Sera were obtained after a 4 °C centrifugation of blood collected in plain tubes (Vacutainer, Becton Dickinson, Rutherford, NJ, USA) stored in iced water (11).

The centrifugation and the separation were performed within 1 hour of blood withdrawal. Sera were stored at −20 °C and were thawed only for analysis, performed contemporarily by two operators. Cell culture media were treated as sera. Sera and culture media stored more than 1 month at −20 °C were discarded because intact osteocalcin became degraded with a 70%–80% loss of immunoreactivity.

Statistics

All the in vivo data obtained with each assay were plotted independently on time on a normal-probability graph and found to lie on a straight line. The goodness of fit was tested by the *Shapiro-Wilk* test, and the distribution was not significantly different from normal. At each time the mean percentage difference of the median from the mean was within 15%. Data were analysed by a one factor ANOVA for repeated measurements and by *Duncan*'s multiple range test. Data from the in vitro study were analysed by a one way ANOVA and by *Duncan*'s multiple range test; basal values versus after treatment values were compared with the *Student*'s "t" test for paired observation. Statistical analysis was performed with UNISTAT, statistical package vs. 3, 1994.

Results

In vivo

After a short course of calcitriol administered daily as the oral dose of 1 µg, all assays showed the same trend: osteocalcin serum levels increased reaching a significant ($p < 0.05$) peak after 8 days from the beginning of the treatment and returned to baseline levels within 30 days (fig. 1, top). Elsa-osteo-nat showed a rise in serum osteocalcin (150%) that was higher than that shown by the EIA (138%) and the OSTK-PR (139%), although it was not statistically significant.

None of the assays revealed any significant rise of osteocalcin concentration during and after the oral treatment with 0.5 µg daily of calcitriol (fig. 1, bottom).

Before and after treatment with both calcitriol doses, the absolute values of osteocalcin concentrations measured with Elsa-osteo-nat were significantly higher ($p < 0.01$) than those measured with the other two assays.

In vitro

Basal values of osteocalcin measured with Elsa-osteo-nat, EIA and OSTK-PR in the culture media of human osteoblast-like cells did not differ and were 0.012 ± 0.001, 0.009 ± 0.003 and 0.011 ± 0.002 g/kg of protein, respectively. After exposure to calcitriol a highly significant ($p < 0.0001$) osteocalcin increase was detected by all the three tested assays. Absolute values of osteocalcin after calcitriol differ significantly ($p < 0.01$) depending on the assays used (fig. 2): Elsa-osteo-nat showed the highest absolute osteocalcin values.

Discussion

The aim of this study was to test whether different assays, based on different antibodies and analytical methods, have different sensitivities in detecting stimulated osteocalcin production by osteoblasts. For this purpose we measured osteocalcin production after stimulation in two different experimental conditions, one in vivo and the other one in vitro.

In the in vivo condition, the ability of the assays to detect the stimulated osteocalcin production would depend not only upon the characteristics of the assays, but also on the amount of calcitriol administered. As no assay detected any significant rise after administration of 0.5 μg it can be speculated that this calcitriol dose was subliminal for the stimulation of osteocalcin production. In contrast, all assays detected a significant rise in osteocalcin production after 1 μg calcitriol, this dose being above the threshold for the effect.

The choice of scale for expression of response to treatment can modify the conclusion as to which assay displays the best sensitivity. By considering that the absolute and relative differences in osteocalcin production in vivo after 1 μg calcitriol were not statistically significant, it can be concluded that all assays displayed similar sensitivity. However, the highest, although not significant, absolute and relative differences in osteocalcin production detected by Elsa-osteo-nat after stimulation cast doubt on this conclusion and suggest that this latter assay is the most sensitive. This suggestion is supported by the in vitro findings. Human osteoblast-like cells in culture, when derived from human trabecular explants as in the present study, provide one of the best techniques

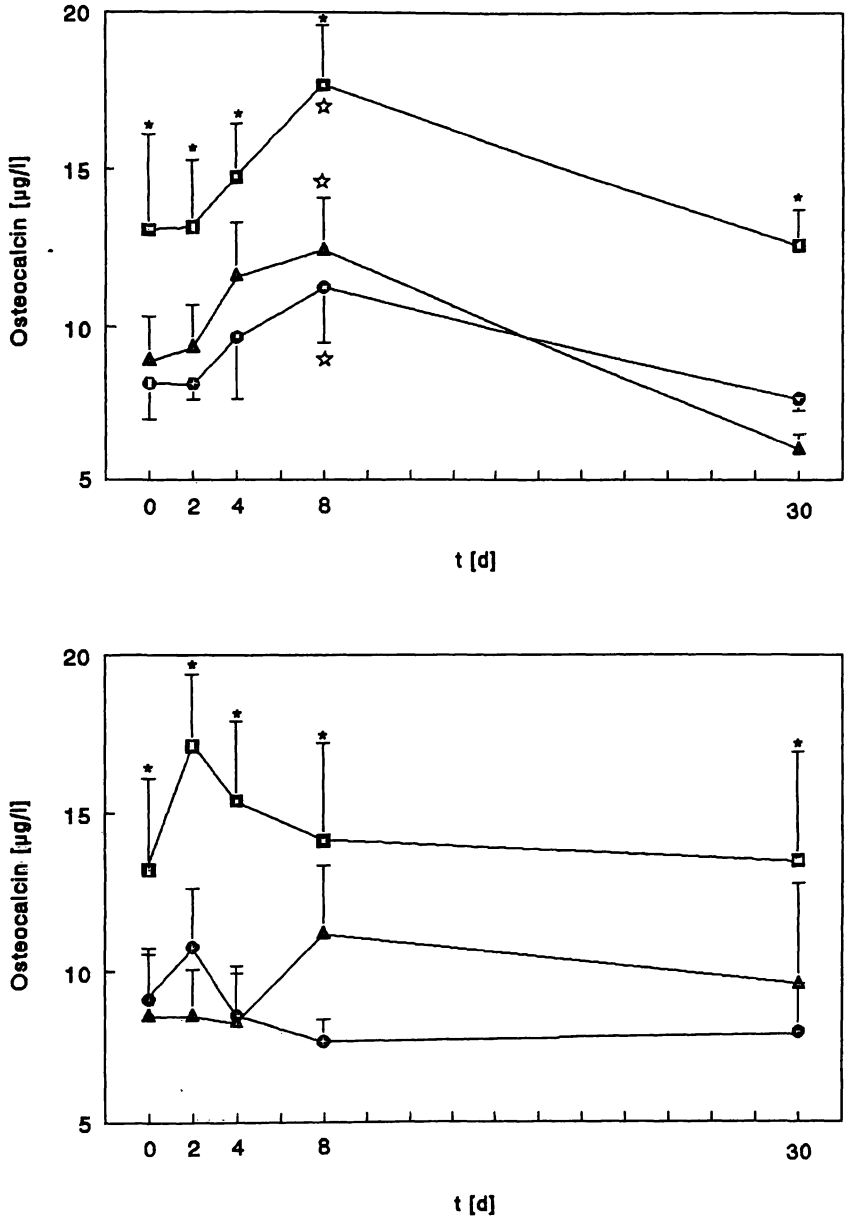

Fig. 1 Osteocalcin concentrations measured with the different assays in sera of osteoporotic women after a short course of calcitriol administered daily at the oral dose of 1 μg (top) and 0.5 μg (bottom). Values are expressed as mean ± SEM (n = 5 in each group). ●: EIA (DAKO); ■: Elsa-osteo-nat (CIS); ▲: OSTK-PR (CIS). *: $p < 0.01$ among assays. ☆: $p < 0.05$ vs t_0 and t_{30}.

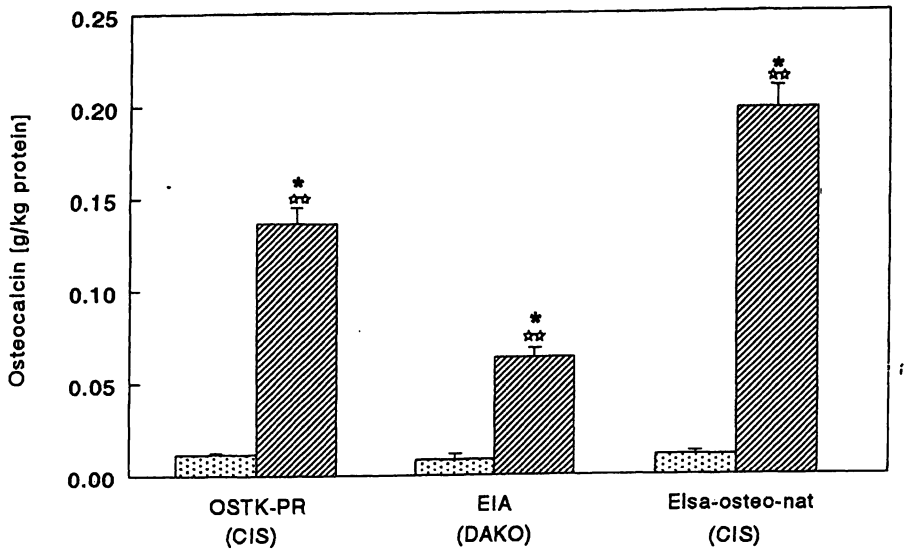

Fig. 2 Osteocalcin concentrations measured in culture medium of human osteoblast-like cells before (basal) and after exposure to 10 nmol/l calcitriol for 48 h. Values are expressed as mean ± SEM (n = 22).
▨: basal; ▨: calcitriol.
*: $p < 0.01$ among assays. ☆☆: $p < 0.0001$ vs basal.

currently available for studies on human bone cell biology (12) and thus represent an optimal challenge for the assays. In contrast to the rat osteosarcoma cell lines, which also produce the peptide in absence of calcitriol (13), osteocalcin production by cells derived from human surgery is absolutely dependent on the presence of calcitriol (14, 15). Under these conditions the assay response is further characterized, and the performance of the Elsa-osteo-nat shown; like the other assays, this assay detected minimal osteocalcin production in the absence of calcitriol, but it detected the highest absolute and relative significant differences in osteocalcin values after calcitriol exposure. Given the absence of significant differences of the basal values measured with the three assays, the higher poststimulus values obtained with Elsa-osteo-nat suggest that this assay is more sensitive than the others in detecting the stimulus.

In vivo, osteocalcin levels measured with Elsa-osteo-nat in serum, either after stimulation or in the basal state, were consistently higher than the absolute values measured by EIA and OSTK-PR. Even though a great variability in osteocalcin levels was observed in the standardization studies, so that certain precautions are advisable in comparing absolute values measured with different assays (16, 17), the higher values detected by Elsa-osteo-nat are surprising if one considers that this is an assay claimed to measure human intact osteocalcin without cross reactivity with osteocalcin fragments. It is possible, however, that the homologous configuration of the assay underlies the higher absolute values detected by Elsa-osteo-nat with respect to the other assays. Higher osteocalcin values have indeed been found with several human homologous assays (18). The better sensitivity of the assay addressed to the intact osteocalcin molecule might also be associated with a better specificity. Indeed, when it was challenged by measuring sera treated with monoclonal antibody against amino acids 43–49 of human osteocalcin, non-detectable values were obtained (19).

Possible underestimation of intact osteocalcin concentrations due to the absence of EDTA in the collecting tubes is unlikely. In fact, the assays were performed immediately after the first thawing, where the advantage of EDTA for osteocalcin preservation was minimal and not statistically significant (20).

In conclusion, the three tested assays displayed similar sensitivity in detecting calcitriol-stimulated osteocalcin production in osteoporotic women with normal glomerular filtration. In vitro, Elsa-osteo-nat showed the best sensitivity. All three tested assays can be applied to serial osteocalcin determination for monitoring the trend of diseases and/or the response to therapy. Only one assay, Elsa-osteo-nat, properly reflects the osteocalcin production by human osteoblasts in culture.

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